AL/EQ-TR-1995-1004

CLONING AND PARTIAL CHARACTERIZATION OF AN ANILINE METABOLIC PATHWAY

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Contract No. F08637-94-M-6013

August 1995

ARMSTRONG LABORATORY

■ **United States Air Force** ■ **Tyndall Air Force Base, FL** ■ **32403-5323**

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
03-AUG-1995	Journal Article -- PREPRINT	01-JAN-1990 -- 01-AUG-1995

4. TITLE AND SUBTITLE

Cloning and Partial Characterization of an Aniline Metabolic Pathway (PREPRINT)

5a. CONTRACT NUMBER

F08637-94-M-6013

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Peretti, Steven W.; Thomas, Stuart M.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

19006102

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Department of Chemical Engineering
North Carolina State University
Raleigh, NC 27695

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Armstrong Laboratory
139 Barnes Drive, Suite 2
Tyndall Air Force Base, FL 32403-5323

10. SPONSOR/MONITOR'S ACRONYM(S)

AL/EQ

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

AL/EQ-TP-95-1004

12. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution A: Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

Submitted to Applied and Environmental Microbiology for publication.

14. ABSTRACT

Previous studies on aniline metabolism determined that the initial enzymatic step removes the amino group and results in formation of catechol. Subsequent cleavage of the ring can occur either via the *ortho* or *meta* pathway. We report here the characterization of a *Pseudomonas sp.*; CIT1, that has the ability to grow on aniline, 3-methylaniline, or 4-methylaniline as the sole carbon and energy source, and cloning of the genes that govern the conversion of aniline to organic acids. The pathway resides on a 20.66 kb BamH1 fragment, and is induced by a broad range of substituted anilines, with para substituted anilines acting as the strongest inducers. The substrate range of the pathway enzymes is also broad, and includes chloro, hydroxy, and methyl substitutions, with preference to additions in the *meta* and para positions.

Metabolism of aniline in CIT1 is initiated by aniline, 1,2 dioxygenase, and results in a stoichiometric release of ammonia and putative formation of catechol. This initial dioxygenation was indicated by quantitive respirometry; aniline required 3.14 moles O_2/mol aniline while catechol requires 2.12 moles O_2/mole catechol. The one mole difference is consistant with a dioxygenation reaction. The ring is cleaved by catechol 2,3 dioxygenase to form a yellow compound with an absorbance maximum at 375 nm, which is consistant with 2-hydroxymuconic semialdehyde. This is further metabolized by an NAD-dependent dehydrogenase and an NAD-independent hydrolase. Aniline metabolism in *E.coli*, expressing the cloned pathway was confirmed using HPLC.

15. SUBJECT TERMS

Cloning, Partial Characterization, Aniline Metabolic Pathway

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Catherine Vogel
U	U	U	SAR	29	19b. TELEPHONE NUMBER *(Include area code)*

Reset

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

Project Summary

We investigated transcriptional regulation of nitroaromatic metabolism in a soil Pseudomonad using aniline (AN) as a model compound. The aniline pathway is induced by a broad range of compounds; exposure to substituted anilines results in the formation of 2 dioxygenases and a suite of meta cleavage enzymes. The size of the fragment containing the operon is 20.66 kilo base pairs and is of comparable size to the lower pathway of toluene metabolism in *P. putida* mt-2, 15.0 kbp. The activity in the recombinant system is approximately 1/30 of the wild type, indicating that this promoter is not efficiently transcribed in *E. coli*. This has been observed for other *Pseudomonas* genes expressed in *E. coli*.

The initial step in aniline metabolism appears to be a dioxygenase attack to form catechol and release ammonia. Presently this claim is only supported by circumstantial evidence as catechol has not been detected in the media. Attempts to cause catechol build-up by inactivation of the catechol 2,3 dioxygenase were not successful as they resulted in the deactivation of the initial enzyme. This implicates an iron binding subunit as is the case with several characterized dioxygenases. We are presently developing a strain that will convert aniline to catechol, allowing us to identify it as an intermediate and also to show that the initial attack is a dioxygenase.

We have subcloned the next enzyme in the pathway, a catechol 2,3 dioxygenase. Analysis of this gene shows that it has homology with *xyl*E from the toluene system. Crude cell extracts from induced cells show that this enzyme has similar activity to purified meta pyrolcatechase II, the substrate range is the same and it is inactivated by iron chelators. SDS PAGE analysis was used to estimate the subunit size to be 35 kilodalton; this compares well to the published size of catechol 2,3 dioxygenase.

The subsequent enzymatic steps result in colorless products and are the result of an NAD-dependent dehydrogenase and an NAD-independent hydrolase. These activities were present in both the wild type and recombinant cell lines. These activities have been well documented in the toluene meta cleavage pathway and have been recently reported in a nitrobenzene pathway.

Based on these results and by comparison with previously characterized aniline pathways and aromatic hydrocarbons, we propose the following pathway: the initial attack is at the 1,2 position to form a aminohyrodiol ,which decomposes to form catechol and release ammonia. Catechol is cleaved to form 2-hydroxy mucconic semialdehyde. Metabolism proceeds through either a dehydrogenase or a hydrolase to TCA cycle intermediates.

Studies are presently underway to subclone the pathway and to fully characterize the operon. Comparison of the broad range amino dioxygenase will be made to other dioxygenases, thus extending the evolutionary analysis of these enzymes. Genetic analysis will be conducted on the promoter and regulatory gene once they have been subcloned. Finally, the broad substrate range will be extended by gene recruitment to allow metabolism of chloroanilines and aminophenols.

The attached manuscript presents our findings in detail, and will be submitted for publication to the professional journal "Applied and Environmental Microbiology".

Cloning and Partial Characterization of an Aniline Metabolic Pathway

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3 August 1995

ABSTRACT

Previous studies on aniline metabolism determined that the initial enzymatic step removes the amino group and results in formation of catechol. Subsequent cleavage of the ring can occur either via the *ortho* or *meta* pathway. We report here the characterization of a *Pseudomonas sp.*, CIT1, that has the ability to grow on aniline, 3-methylaniline, or 4-methylaniline as the sole carbon and energy source, and cloning of the genes that govern the conversion of aniline to organic acids. The pathway resides on a 20.66 kb BamH1 fragment, and is induced by a broad range of substituted anilines, with para substituted anilines acting as the strongest inducers. The substrate range of the pathway enzymes is also broad, and includes chloro, hydroxy, and methyl substitutions, with preference to additions in the *meta* and para positions.

Metabolism of aniline in CIT1 is initiated by aniline 1,2 dioxygenase, and results in a stoichiometric release of ammonia and putative formation of catechol. This inital dioxygenation was indicated by quantitive respirometry; aniline required 3.14 moles O_2/mol aniline while catechol requires 2.12 moles O_2/ mole catechol. The one mole difference is consistant with a dioxygenation reaction. The ring is cleaved by catechol 2,3 dioxygenase to form a yellow compound with an absorbance maximum at 375 nm, which is consistant with 2-hydroxymuconic semialdehyde. This is further metabolized by an NAD-dependent dehydrogenase and an NAD-independent hydrolase. Aniline metabolism in *E.coli*, expressing the cloned pathway was confirmed using HPLC.

INTRODUCTION

Xenobiotic and recalcitrant compounds have been introduced into the environment as a result of human activities, with many of these compounds being either mutagenic or carcinogenic. An important class of environmental pollutants are amino substituted aromatics (anilines) due to their extensive use in the dye, pesticide, plastic, and pharmaceutical industries. Also, they are metabolites resulting from microbial transformation of nitroaromatics such as explosives and herbicides. This has resulted in extensive environmental contamination by ring-substituted anilines and has led to their classification as priority pollutants by the US EPA. As such, efficient and efficacious treatment approaches must be developed.

Biological based systems have the potential to be cost effective for various applications; two examples are reclamation of contaminated soil and destruction of pollutants present in process streams at dilute concentrations. Fundamental understanding of the metabolic pathway is needed to establish that degradation leads to non-toxic products and for the design and operation of treatment systems. Presently, few metabolic systems have been elucidated to the extent needed to maximize the potential of bioremediation as a treatment technology [9; 10; 24; 27]. To further understand amino aromatic metabolism, we have chosen to focus on aniline metabolism, and report here the characterization of this pathway found in a *Pseudomonas sp.* CIT1[17] and the cloning of these metabolic genes into *E.coli.*

Many reports of aniline utilization as the sole carbon and energy source have been published[1; 2; 4; 17; 26], with substrate inhibition observed between 1500-2600 ppm depending on the bacterium, [1; 2; 11]. The ability to utilize substituted anilines appears to be strain dependent. *Pseudomonads* and *Moraxella* have been shown to use some chloro- and methyl-anilines [21; 28; 29], while *Rhodococcus* show limited substrate range [1; 2].

Analysis of the degradation products have resulted in postulated pathways for the conversion of aniline and substituted anilines to TCA cycle intermediates. In all reported cases the initial reaction is formation of catechol with release of ammonia; this is predominantly followed by *ortho* ring cleavage [1; 2; 29]. The bias towards this route may be a result of the

exposure history of the site from which the bacterium was isolated. Many of the industrially useful anilines contain a chloro substitution. *Ortho* cleavage of chloroaromatics results in energy generating intermediates, while *meta* cleavage results in the formation of nonproductive ("dead-end") or highly reactive ("suicide") products that ultimately lead to cell death [22]. In contrast, the predominant route for methyl anilines appears to be the *meta* cleavage pathway[3; 13].

Although pure cultures have been identified and biochemical pathways elucidated, few reports on the genetics of aniline catabolism exist [17]. We report here the characterization of aniline metabolism in a *Pseudomonas sp.* and cloning of genes that govern the catabolism. The ring cleavage gene has been sub-cloned and is a catechol 2,3 dioxygenase. The substrate range of the pathway enzymes is broad and includes methyl, hydroxyl, and chloro substitutions; however, the growth substrate range is limited to aniline and methyl aniline(3&4). Expression of catechol 2,3 dioxygenase is positively regulated in recombinant *E. coli* containing the cloned pathway and is induced by a broad range of compounds.

MATERIAL AND METHODS

Bacterial strains and plasmids. The Pseudomanas strain CIT1 was isolated from soil by its ability to grow on aniline as the sole source of carbon and energy. It has been shown that the aniline genes are contained on a 100 kb plasmid [17]. Cloning experiments were conducted using the strain *E. coli* DH5α as the host. Its geneotype is ϕ80dlacZΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17, supE44, thi-1, gyrA96(nalidixic acid resistant), relA1. The deletion in lacZ can be complemented by pUC18 to produce functional enzyme.

The plasmids used in this study are: pUC18, pVDX13[14], pXYLE, pSMT3, pSMT4. All of these contain 1 copy of the β-lactamase gene that confers resistance to ampicillin. The plasmids pXYLE, pSMT3, and pSMT4 are pUC18 based products of this study and their construction is explained in the materials and methods section. Plasmid pSMT3 contains a 5.4 kb fragment of the aniline pathway from *P. sp.* CIT1 and expresses catechol 2,3 dioxygenase from the lactose promoter. Plasmid pSMT4 contains a 20.66 kb fragment from *P. sp.* CIT1 and

confers the ability to convert aniline to organic acids. Plasmid pXYLE contains the xylE gene of the TOL pathway and expresses catechol 2,3 dioxygenase from the lactose promoter.

Media and chemicals. L-salts media was prepared as follows: NH_4Cl 5%, 5mL/L; $CaCl_2$ 1.5%, 1mL/L; $FeSO_4$ 0.1%, 1mL/L; $MgSO_4$ 20%, 1mL/L; $NaNO_3$ 20%, 5mL/L; KCl 4%, 1mL/L; Na_2HPO_4 2.1%, NaH_2PO_4 0.9%; 10mL/L; MoO_3 0.1%, 1mL/L. Trace elements contain: $CuSO_4 \cdot 5H_2O$ 0.00005%, H_3BO_3 0.1%, $MnSO_4 \cdot 5H_2O$ 0.1%, $ZnSO_4 \cdot 7H_2O$,1mL/L. The pH of the media is adjusted to 7.2 and sterilized by autoclaving for 25 minutes. Agar plates were prepared by adding 15g/L of DIFCO bacto-agar before autoclaving. Aniline, 99.5% Aldrich Chemicals (Milwaukee, WI) was added directly to the media following sterilization.

Chemicals for the substrate range experiments were purchased from Aldrich Chemicals (Milwaukee, WI) and were of the highest purity available. 2-hydroxymuconic semialdehyde (HMS) was prepared from catechol using crude cell extracts from DH5α/pXYLE.

Restriction and DNA modification enzymes were obtain from Promega(Madison WI).

Analytical Techniques HPLC analysis was performed using an Econosphere C_8 column (Alltech, Deerfield, Ill) with a mobile phase of water and methanol each containing 0.1% trifluoroacetic acid. The elution profile was 100% water for 3.7 minutes, at 3.8 minutes the composition was switched to 5% water and 95% methanol, and held at this ratio for 6.2 minutes. The compounds were monitored with a Spectra-Physics (Piscataway, NJ) Spectra Chrom 200 UV detector set to 254 nm and the flow rate was 1.5 mL/min.

Total protein and ammonia levels were determined using standard assays, the kits were purchased from Sigma Chemical Company (St. Louis, MO). Bovine serum albumin was used to construct a standard curve for the protein determination. Ammonia was determined by following the oxidation of NADH in the presence of oxogluterate and L-glutamate dehydrogenase.

Aniline concentration was determined using a correlation obtained from standard solutions prepared in 20mM sodium phosphate pH 7.5. The absorbance at 278 nm was measured on a Shimadzu uv160u scanning spectrophotometer. If needed, experimental samples were diluted with sodium phosphate (pH 7.5).

SDS PAGE was performed using the method of Laemmli [15].

Enzyme assays. Catechol 2,3-dioxygenase was assayed according to the method of Kojima *et al.*[16]. 2-hydroxymuconic semialdehyde dehydrogenase and 2-hydroxymuconic semialdehyde hydrolase activities were measured spectrophotometrically by following the decrease in absorbance at 375 nm. Both HMS dehydrogenase and HMS hydrolase activities were measured in reaction mixtures containing dialyzed cell extract, HMS and 100 μM of NAD. HMS hydrolase activity was measured using only the dialyzed cell extract and HMS. The difference between these two activities was attributed to HMS dehydrogenase activity.

Recombinant DNA Techniques. The isolation of total genomic DNA was reported previously [8]. Manufacturers protocols were followed for all enzyme reactions. Plasmid DNA was isolated as previously described[5].

Substrate range studies. Chemostat grown *P. sp.* CIT1, with aniline as the sole carbon source, were spun down in a Sorvall SS34 rotor, 7000 rpm at 4 ^{o}C for 10 minutes. The pellets were washed two times with 20 mM sodium phosphate pH 7.0. The cells were resuspended to 1 O.D. unit at 600 nm in phosphate buffer. These were placed on ice and aerated to reduce background oxygen consumption. Oxygen uptake experiments were performed using a YSI Inc. model 5300 biological oxygen monitor (Yellowsprings, Ohio). The sample cuvettes contained 3mL of cells and putative substrates were added at 166 μM.

Growth range studies. *Pseudomonas sp.* CIT1 was grown on L-salts minimal media with various substituted anilines (125ppm) as the sole carbon source. The inoculum was *P. sp.* CIT1 grown on LB-agar plates overnight at 30 ^{o}C. Growth was determined by following the O.D. at 600nm using a Shimadzu uv 160u scanning spectrophotometer.

Induction studies. Recombinant *E. coli* strain DH5α/pSMT4 was grown on L-salts with glucose(0.4%) and thiamine-HCl (0.166 μg/mL). Various substituted anilines were added as inducers; the final concentration of each was 50 mg/L. Shake flasks were inoculated with cells grown on LB agar plates and were grown overnight in a temperature controlled shaker at 37 ^{o}C and 250 rpm. The O.D. at 600nm of the culture was taken after inoculation and just prior to

harvesting. The specific activity of catechol 2,3 dioxygenase was used as the measure of induction.

Aniline metabolism and ammonia release. *P. sp.* CIT1 cultivated in continuous culture in a chemostat was spun down in a Sorvall SS 34 rotor 7000 rpm at 4 ^{o}C. The cell pellet was washed twice with 20mM sodium phosphate pH 7.0. The cells were resuspended in 100 mLs of phosphate buffer and 15 uL of aniline was added. Samples were aseptically removed, the cells were removed by a 7 minute spin in a microfuge, 15,000 rpm. The supernatant was placed at -40 ^{o}C until the ammonia levels were assayed. An aliquot of the supernatant was used to determine the aniline concentration.

Cloning of xylE, andioxB and the aniline pathway. The xylE gene of the TOL pathway was removed from the promoter probe vector pVDX13 using the restriction endonuclease Sac1 and the 1.8 kb fragment was ligated into the multiple cloning site of pUC18. This plasmid was named pXYLE1. Recombinant cells that express this gene turn bright yellow when exposed to catechol. Cell extracts from this strain were used as the positive control for SDS PAGE electrophoresis. This strain was also used to make 2-hydroxymuconic semialdehyde from catechol.

A 5.4 kb fragment of the aniline pathway was cloned from a total genomic DNA preparation from *P. sp.* CIT1. The isolated DNA was cut with restriction endonuclease EcoR1. The restricted DNA was ligated into the EcoR1 site of pUC18 with T4 DNA ligase. *E. coli* DH5α was electroporated with the ligation mix using a Bio Rad Gene Pulser with a pulse controller using the high efficiency protocol of the manufacturer. The cells were transferred to SOC [18] and grown at 37 ^{o}C for one hour prior to plating on LB-ampicillin (75 μg/mL). The plates were incubated for 40 hours at 37 ^{o}C. Clones that expressed the catechol 2,3 dioxygenase gene turned bright yellow when the plates were sprayed with 300mM sterile catechol. This gene was named andioxB.

Restriction enzyme analysis of andioxB indicated that neither BamH1 or HindIII restriction sites exist in this fragment. Therefore, Southern analysis of genomic DNA restricted

with BamH1 was conducted [18]. The non-radioactive BioNick labeling and Blugene detection kits from Gibco BRL(Gaithersburg, MD) were used to label the probe (andioxB) and to detect the hybrid, a 20.66 kb fragment. A preparative DNA digestion with BamH1 was performed and fractionated in a 5-20% potassium acetate gradient using a Sorval TH 641 swinging bucket rotor operated at 27,000 rpm for 10 hours at 10 ^{o}C. Gel electrophoresis of ethanol-precipitated aliquots was performed to identify fractions that contained DNA in the size range of 18-25 kb pairs; these were saved for cloning experiments. Ultra purified cloning vector pUC18 was restricted with BamH1 and dephosphatased with calf intestine phosphatase, Promega (Madison,WI). DNA obtained from the size fractionation procedure was ligated into pUC18 with T4 DNA ligase. Cells that contained the recombinant plasmid were selected on LB-amp(75mg/mL) and 20ug/mL of X-gal. White colonies were picked and transferred to microtiter plates containing LB-amp and grown overnight at 30 ^{o}C. These colonies were then transferred to fresh microtiter plates that contained L-salts, thiamine-HCl (0.166μg/mL), glucose(0.4%), and aniline (50ppm), and allowed to grow overnight at 30 ^{o}C. The plates were assayed by adding 10 ul of sterile 800ppm catechol to each well. Wells that turned yellow were found to contain clones that expressed the aniline metabolic genes. The 5.4 kb andioxB fragment was recovered from the larger 20.66 kb BamH1 insert. The plasmid containing the aniline pathway was named pSMT4.

Aniline metabolism by recombinant *E. coli*. DH5α/pSMT4 were grown in L-salts, glucose(0.4%), aniline(70 ppm), and thiamine-HCl. The flasks were inoculated with cells from LB-amp agar plates and grown at 37 ^{o}C in a rotary shaker. The negative control was DH5α/pUC18 grown on the same media. The flasks were sampled after 18 hours and metabolism of aniline was determined by HPLC.

RESULTS

Cloning of catechol 2,3 dioxygenase from the aniline pathway. An important step in the catabolism of aromatics is the cleavage of the ring to form organic acids. *Pseudomonas sp.* CIT1 grown on aniline exhibits high levels of the *meta* cleavage enzyme catechol 2,3 dioxygenase, when compared to cultures with either succinate or yeast extract as the carbon source. This is shown in Table 1 and Figure 1. The DNA fragment containing the andioxB gene has homology to xylE as determined by Southern analysis, data not shown. SDS page analysis of cell extract from DH5α/pSMT3 shows a 35 kilodalton band. No band appears in the control, DH5α/pUC18, at this molecular weight. Cell extract from the positive control, DH5α/pXYLE, also contains a protein band at approximately 35 kDa, the published size of the catechol 2,3 dioxygenase monomer [16], as indicated in Figure 1. These results show that a catechol dioxygenase is present in the aniline metabolic pathway and suggests that aniline is converted to catechol with subsequent *meta* ring cleavage.

Cloning of the genes in the aniline metabolic pathway. Southern analysis of DNA from *P. sp.* CIT1 indicates that a 20.66 kb BamH1 fragment has homology to the andioxB fragment previously isolated from this strain, as shown in Figure 2. This fragment was cloned into pUC18 to make pSMT4. Plasmid DNA from DH5α/pSMT4 was characterized by gel electrophoresis. The 5.4 kb EcoR1 fragment was recovered along with 2 more EcoR1 fragments of approximately the same size, as indicated in Figure 3. Metabolism of aniline by this strain was confirmed by HPLC analysis of media from overnight cultures. The recombinant strain degraded approximately 90 percent of the aniline while the control flask contained 95 percent of the initial aniline, as indicated in Figure 4. However, aniline was not used as the sole source of carbon and energy by the recombinant strain. Instead, glucose was supplied for this function.

Growth range of *P. sp.* CIT1. Shake flask studies show that this strain can use aniline and 3- and 4-methylaniline as carbon and energy sources, while hydroxy and chloro substituted anilines failed to stimulate growth. Growth was determined by measuring the change $O.D._{600}$ over three

days. Cultures with compounds that stimulated growth had a final $O.D._{600}$ of approximately 0.137, while the $O.D._{600}$ fell to approximately zero in all of the other shake flasks.

Substrate range of aniline pathway enzymes. Oxygen uptake was stimulated by a wide range of substituted anilines and methyl catechols, as shown in Table 3. 3- or 4- toluidine and 3-aminophenol resulted in uptake rates that were 25% that of aniline. 4-chloroaniline and 4-aminophenol resulted in even lower uptake rates, approximately 10% that of aniline. All other compounds tested had uptake rates that were substantially lower still. Aniline did not stimulate oxygen uptake in cells grown in shake flasks with succinate as the carbon source. An interesting aside is that 4-nitrophenol (PNP) elicited oxygen uptake in both aniline and succinate cultured cells. The rate was 0.045 and 0.0772 $\mu M/s\text{-}O.D._{600}$ for aniline and succinate cultured cells respectively. Suggesting the presence of a constitutive PNP pathway in this organism.

Several substituted catechols were assayed to determine the substrate specificity of the catechol 2,3 dioxygenase from *P. sp.* CIT1. 3- and 4-methylcatechol stimulated oxygen uptake at 33% and 66% that of catechol respectively, and 1,2,4 benzenetriol resulted in a 20% uptake rate with respect to catechol. No response was elicited by the addition of protocatechuate, 4-nitrocatechol, phenol or hydroquinone. These results are consistent with catechol dioxygenases from other strains [16; 20]. Activities of uninduced cells were very low, approximately 3% of the induced cells, and showed the same relative activities toward the substrates. 1,2,4 benzenetriol was the exception to this result with uninduced cells maintaining 11% of their activity. This suggests that another enzyme may be present that can metabolize 1,2,4 benzenetriol.

Pathway induction. The response of the pathway to addition of various inducers is shown in Table 2. The general trend was that substitution in the four position stimulates greater pathway activity than in the three position. Both 4-chloro and 4-methyl aniline elicited higher induction levels than did aniline. Glucose alone resulted in catechol 2,3 dioxygenase levels that were 21% of an aniline induced culture, indicating that catabolite repression by glucose is not an issue. The presence of low constitutive levels of the reporter enzyme in the clones mimics the regulatory

behavior of the wild type, i.e. basal levels of catechol 2,3 dioxygenase when *P. sp.* CIT1 are cultured on L-salts with 0.2% yeast extract or 0.4% succinate (Table 1).

Aniline metabolism and ammonia release. The stoichiometry of ammonia production and aniline metabolism was determined to be approximately 1:1, as shown in Figure 5. Results from quantitative respirometry show oxygen consumption of 3.14 and 2.12 mol/mol for aniline and catechol respectively. The 1 mole difference is consistent with a dioxygenase attack on aniline to form catechol. Sequential monooxygenase attacks would require 2 moles of oxygen to produce catechol and the induced cells show no activity toward phenol. These results, and the high levels of catechol 2,3 dioxygenase in aniline cultivated cells, suggest that the amino group is removed by a dioxygenase to form catechol. The basal constitutive level of catechol 2,3 dioxygenase in uninduced cells precluded the accumulation of catechol in the media. Also, inactivation of catechol 2,3 dioxygenase with 1,10-phenanthroline or 2, 2'-Dipyridyl [16] resulted in the deactivation of aniline 1,2 dioxygenase; it was therefore not possible to identify catechol in the cultures.

Metabolism of 2-hydroxymuconic semialdhyde, HMS. Metobolism of HMS has been shown to proceed through two mechanisms; 1) an NAD-dependent dehydrogenase and 2) an NAD-independent hydrolase. These are both present in various organisms and both are present in aniline grown *P. sp.* CIT1. It was necessary to remove residual NAD from the fresh crude cell extract. This was accomplished by dialyzing for 2 hours against two changes of sodium phosphate buffer (pH 7.0, 20 mM), shown by Nishino *et al.* to be an effective method to remove NAD and to determine these enzymatic activities [20]. The difference between activities in reactions with and without NAD indicated that dehydrogenase was present. There was an increase of 22% in the initial slope when NAD(100μM) was included in the reaction mixture.

DISCUSSION

Aniline and substituted anilines have made their way into the environment through many routes, resulting in bacteria that have developed metabolic pathways to take advantage of this occurrence. The specific pathway depends on the exposure history of the microorganisms; cells from chloroaniline-contaminated sites use *ortho* ring cleavage while cells capable of metabolizing methlyanilines employ the *meta* cleavage pathway. Therefore, use of this strain is limited to a narrow range of compounds unless genetic manipulation or mutagenesis is used to extend the growth range.

The aniline pathway is induced by a broad range of compounds; exposure to substituted anilines results in the formation of 2 dioxygenases and a suite of *meta* cleavage enzymes. The cloned pathway is 20.66 kb and is of comparable size to the lower pathway of toluene metabolism in *P. putida* mt-2, (15.0 kb). The activity in the recombinant system is approximately 1/30 of the wild type, indicating that this promoter is not efficiently transcribed in *E. coli*. This has been observed for other *Pseudomonas* genes expressed in *E. coli* [7]. The sigma factor requirement has been implicated as the primary source of the poor transcription rate. *E. coli* generally uses sigma 70 while some *Pseudomonas* promoters require sigma 54, which is not commonly used in *E. coli*. [25]. Subcloning and sequencing would make available the first comparison of amino aromatic promoters and regulatory genes with previously characterized systems [6; 12; 19].

The initial step in aniline metabolism appears to be a dioxygenase attack to form catechol and release ammonia; we have labeled the putitive initial enzyme aniline 1,2 dioxygenase. Presently this claim is only supported by circumstantial evidence as catechol has not been directly detected in the media. Attempts to cause catechol build-up by inactivation of the catechol 2,3 dioxygenase with common iron cheletors were not successful, as they resulted in the deactivation of aniline 1,2 dioxygenase. This implies the presence of an iron binding subunit in this enzyme, as is the case with several characterized dioxygenases [16; 23]. We are presently

subcloning andioxA to develop a strain that will convert aniline to catechol. This would allow us to identify it as an intermediate and to show directly that the initial attack is a dioxygenase.

Aniline 1,2 dioxygenase exhibits activity toward a broad range of anilines with preference toward nonpolar substitutions. Chloro and hydroxyl substituents can be processed if they are present in the three or four position, while a carboxylic acid addition, to even the four position, results in no activity. This suggests that two mechanisms govern the overall activity of this enzyme; first is position of substitution which most likely envolves steric conciderations at the active site. Substitutions to the three and four position are preferred, and 2-methylaniline is the only compound with an *ortho* substitution that exhibites substantial enzymatic activity. Second, increasing polarity of the substituent results in decreasing enzyme activity, and suggests that interactions between the active site of the enzyme and the substrate are effected by hydrogen bonding. The enzyme most likely posseses a hydrophobic pocket that to some extent can accommodate polar substrates.

We have subcloned the next enzyme in the pathway, a catechol 2,3 dioxygenase. Analysis of this gene shows that it has homology with xylE from the TOL system. Crude cell extracts from induced cells show that this enzyme has similar activity to purified *meta* pyrolcatechase II [16]. The substrate range is similar and it is inactivated by iron chelators. SDS page analysis was used to estimate the subunit size to be 35 kilodalton; this compares well to the published size of catechol 2,3 dioxygenase.

The subsequent enzymatic step results in a colorless product and is the result of an NAD-dependent dehydrogenase and an NAD-independent hydrolase. Both enzymes were present in the wild type and recombinant cell lines. This activity has been well documented in the toluene *meta* cleavage pathway[9] and has been recently reported in a nitrobenzene pathway [20].

The ability of *P. sp.* CIT1 to grow on selected substrates is governed by availability of the necessary enzymes. Compounds that are capable of supporting growth must both turn-on the pathway and be metabolized to energy generating compounds. We have determined that all of the substituted anilines examined elicited, to some extent, transcription of the pathway.

However, methylanilines were the only family of compounds that supported growth of the organism. The chloroanilines efficiently induced expression of the pathway enzymes, but most likely result in intermediates that are not completely metabolized. Whereas the aminophenols are accepted as pathway substrates at levels that should support growth, they do not effectively induce formation of the enzymes. The induction studies with 3-aminophenol showed that the pathway was induced only 16% above basal levels. Therefore, expansion of the growth substrate range of this organism would require both enzyme recruitment and mutagenesis of the regulatory protein to accept a wider range of compounds.

Based on these results, and by comparison with previously characterized aniline pathways and aromatic hydrocarbons, we propose the pathway presented in Figure 6. The initial attack is at the 1,2 position to form a aminohyrodiol which proceeds to catechol with a release ammonia. Catechol is cleaved to form 2-hydroxymucconic semialdehyde. Metabolism proceeds through a dehydrogenase and hydrolase to TCA cycle intermediates.

Studies are presently underway to subclone the pathway and to fully characterize the operon. Comparison of the broad range amino dioxygenase will be made to other dioxygenases, thus extending the evolutionary analysis of these enzymes. Genetic analysis will be conducted on the promoter and regulatory gene once they have been subcloned. Finally, the substrate range will be extended by gene recruitment to allow metabolism of chloroanilines and aminophenols.

ACKNOWLEDGEMENTS

We would like to thank Dr. Philip Warner for providing the strain *P. sp.* CIT1. We thank Thomas K. Wood and Jennifer L. Goodall for suggestions and thoughtful discussion throughout this work. We thank S. Q. Goodall for photographic assistance.

This work was supported by AFOSR and the NSF.

REFERENCES

1. **Aoki, K., et al.** 1983. Isolation of aniline-assimilating bacteria and physiological characterization of aniline biodegradation in Rhodococcus erythropolis AN-13. Agric. Biol. Chem. **47:** 2569-2575.

2. **Aoki, K., R. Shinke, and H. Nishira.** 1983. Metabolism of aniline by Rhodococcus erythropolis AN-13. Agric. Biol. Chem. **47:** 1611-1616.

3. **Appel, M., T. Raabe, and F. Lingens.** 1984. Degradation of o-toluidine by *Rhodococcus rhodochrous*. FEMS Microbiol. Lett. **24:** 123-126.

4. **Bachofer, R., F. Lingens, and W. Schafer.** 1975. Conversion of aniline into pyrocatechol by Nocardia sp.;incorporation of oxygen-18. FEBS Letters. **50:** 288-290.

5. **Birnboim, H., and J. Doly.** 1979. A Rapid Alkaline Extraction Procedure For Screening Recombinant Plasmid DNA. Nucleic Acids Research. **7:** 1513-1523.

6. **Deretic, V., et al.** 1989. Common Denominators of Promoter Control in Pseudomonas and Other Bacteria. Bio/Technology. **7:** 1249-1254.

7. **Franklin, F. C. H., et al.** 1981. Molecular and fucntional analysis of the TOL plasmid pWWO from Pseudomonas putida and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. U.S.A. **78:** 7458-7462.

8. **Goldberg, J. B., and D. E. Ohman.** 1984. Cloning and Expression in *Pseudomonas* aeruginoosa of a Gene Involved in the Production of Alginate. Journal of Bacteriology. **158:** 1115-1121.

9. **Harayama, S., P. R. Lehrbach, and K. N. Timmis.** 1984. Transposon mutagenesis analysis of meta-cleavage pathway operon genes of the TOL plasmid of Pseudomonas putida mt-2. J. Bact. **160:** 251-255.

10. **Harayama, S., et al.** 1989. Characterization of five genes in the upper-pathway operon of TOL plasmid pWWO from *Pseudomonas putida* and identification of gene products. J. Bacteriol. **171:** 5048-5055.

11. **Helm, V., and H. Reber.** 1979. Investigation on the regulation of aniline utilization in Pseudomonas multivorans strain An 1. European J. Appl. Microbiol. Biotechnol. **7:** 191-199.

12. **Inouye, S., et al.** 1986. Nucleotide Sequence of a DNA Segment Promoting Transcription in *Pseudomonas putida.* J. Bact. **166:** 739-745.

13. **Konopka, A.** 1993. Isolation and characterization of a subsurface bacterium that degrades aniline and methylanilines. FEMS Microbiol. Lett. **111:** 93-100.

14. **Konyecsni, W. M., and V. Deretic.** 1988. Gene. **74:** 375.

15. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. **227:** 680-685.

16. **Lidstrom, M. E.** 1990. Hydrocarbons and Methylotrophy. San Diego: Academic Press, Inc.

17. **Mackinnon, G., and J. Anson.** 1984. Novel *Pseudomonas* Plasmid Involved in Aniline Degradation. Appl. Environ. Microbiol. **48:** 868-869.

18. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Labratory.

19. **Mermod, N., et al.** 1984. Transcription of the TOL plasmid toluate catabolic pathway operon of pseudomonas putida is determined by a pair of co-ordinately and positively regulated overlapping promoters. EMBO, J. **3:** 2461-2466.

20. **Nishino, S. F., and S. J. C.** 1995. Oxidative pathway for the biodegradation of nitrobenzene by strain JS765. Applied and Environmental Microbiology. **61:** 2308-2313.

21. **Reber, H., V. Helm, and N. G. K. Karanth.** 1979. Comparative studies on the metabolism of aniline and chloroaniline by Pseudomonas multivorans strain An 1. European J. Appl. Microbiol. Biotechnol. **7:**181-189.

22. **Rojo, F., et al.** 1987. Assemblage of Ortho Cleavage Route for Simultaneous Degradation of Chloro- and Methylaromatics. Science. **238:**1395-1398.

23. **Suen, W., and D. T. Gibson.** 1993. Isolation and preliminary characterization of the subunits of the terminal component of naphthalene dioxygenase from *Pseudomonas putida* NCIB 9816-4. J. Bacteriol. **175:**5877-5881.

24. **Suen, W. C., and J. C. Spain.** 1993. Cloning and Characterization of Pseudomonas sp. Strain DNT Genes 2,4-Dinitrotoluene Degradation. J. Bact. **175:**

25. **Thomas, C. M.** 1989. Promiscuous Plasmids of Gram-Negative Bacteria. San Diego: Academic Press, Inc.

26. **Walker, N., and D. Harris.** 1969. Aniline utilization by a soil Pseudomonad. J. Appl. Bact. **32:** 457-462.

27. **Yen, K., and I. C. Gunsalus.** 1982. Plasmid gene organization: Naphthalene/salicylate oxidation. Proc. Natl. Acad. Sci. USA. **79:** 874-878.

28. **You, I.-S., and R. Bartha.** 1982. Metabolism of 3,4-Dichloroaniline by *Pseudomonas putida*. J. Agric. Food Chem. **30:** 274-277.

29. **Zeyer, J., A. Wasserfallen, and K. Timmis.** 1985. Microbial minerilization of ring-substituted anilines through ortho-cleavage pathway. Appl. Environ. Microbiol. **50:**447-453.

Table 1. Induction of the aniline metabolic pathway in *E. coli* DH5α and *P. sp.* CIT1

INDUCER	*E. coli* DH5α/pSMT4[a] Catechol 2,3 dioxygenase sp act (nmol/ min-μg protein)	PERCENT
Aniline	0.075	100
4-Chloroaniline	0.099	132
3-Chloroaniline	0.047	63
4-Methylaniline	0.086	116
3-Methylaniline	0.049	65
3-Aminophenol	0.028	37
Glucose	0.016	21
INDUCER	*P. sp.* CIT1 Catechol 2,3 dioxygenase sp act (nmol/ min-μg protein)	PERCENT
Yeast Extract[b]	0.016	NA
Succinate[b]	0.009	NA
Aniline[c]	2.5	NA

[a] Cultures were grown overnight at 37 °C in flasks containing 0.4% glucose, 0.166 μg/ml Thiamine-HCl, and 50 ppm of each inducer.

[b] Cultured at 30 °C in shake flasks with 0.4% succinate or 0.2% yeast extract

[c] Chemostat cultured, 2000 ppm aniline inlet concentration.

Table 2. Oxygen uptake by washed suspensions of *P. sp.* CIT1

Assay substrate[a]	Oxygen uptake, μM/s-O.D.
Aniline	0.603 (0.0)
2-Chloroaniline	0.0
3-Chloroaniline	0.009
4-Chloroaniline	0.075 (0.0)
2-Aminophenol	0.004
3-Aminophenol	0.159
4-Aminophenol	0.062 (0.0)
2-Toluidine	0.132
3-Toluidine	0.181 (0.0)
4-Toluidine	0.161
PABA	0.0
Catechol	1.320 (0.040)
3-Methylcatechol	0.431 (0.018)
4-Methylcatechol	0.875 (0.026)
1,2,4 Benzenetriol	0.259 (0.030)
Protocatechuic Acid	0.0
4-Nitrocatechol	0.0
4-Nitrotoluene	0.0
Phenol	0.0
Toluene	0.0

[a] Substrate concentration, 170 μM.

[b] Values are given for chemostat grown cell with aniline as the carbon source, and values in parentheses are for succinate-grown cells.

FIGURE CAPTIONS

Figure 1. SDS-PAGE anaysis of crude enzyme extracts. Lanes were loaded as follows, 1) *P. sp.* CIT1 aniline grown, 2) *P. sp.* CIT1 succinate grown, 3) Molecular Weight Marker,4) DH5α/pSMT3 with IPTG, 5) DH5α/pSMT3 without IPTG, 6) DH5α/pUC18, 7)DH5α/pXYLE, 8)Molecular Weight Marker .

Figure 2. Southern analysis of *P. sp.* CIT1 DNA. Lanes were loaded as follows, 1) Lambda DNA restricted with Hind III, 2) *P. sp.* CIT1 DNA restricted with BamH1, 3) Lambda DNA restricted with Hind III, 4) *P. sp.* CIT1 DNA restricted with EcoR1, 5) pSMT3 DNA restricted with EcoR1, 6) Lambda DNA restricted with Hind III.

Figure 3. Horizontal gel electrophoresis of recombinant *E. coli.* Lanes were loaded as follows, 1) Lambda DNA restricted with Hind III, 2) pUC18 restricted with BamH1, 3) pSMT restricted with EcoR1, 4) Lambda DNA restricted with Hind III 5) pSMT4 restricted with EcoR1, 6) pSMT4 restricted with EcoR1 and BamH1, 7) pSMT4 restricted with BamH1, 8) Lambda DNA restricted with Hind III.

Figure 4. HPLC analysis of aniline metabolism by cultured on aniline and glucose A) DH5α/pUC18 and B) DH5α/pSMT4.

Figure 5. Aniline metabolism and ammonia release by *P. sp.* CIT1.

Figure 6. Proposed pathway for aniline metabolism in *P. sp.* CIT1.

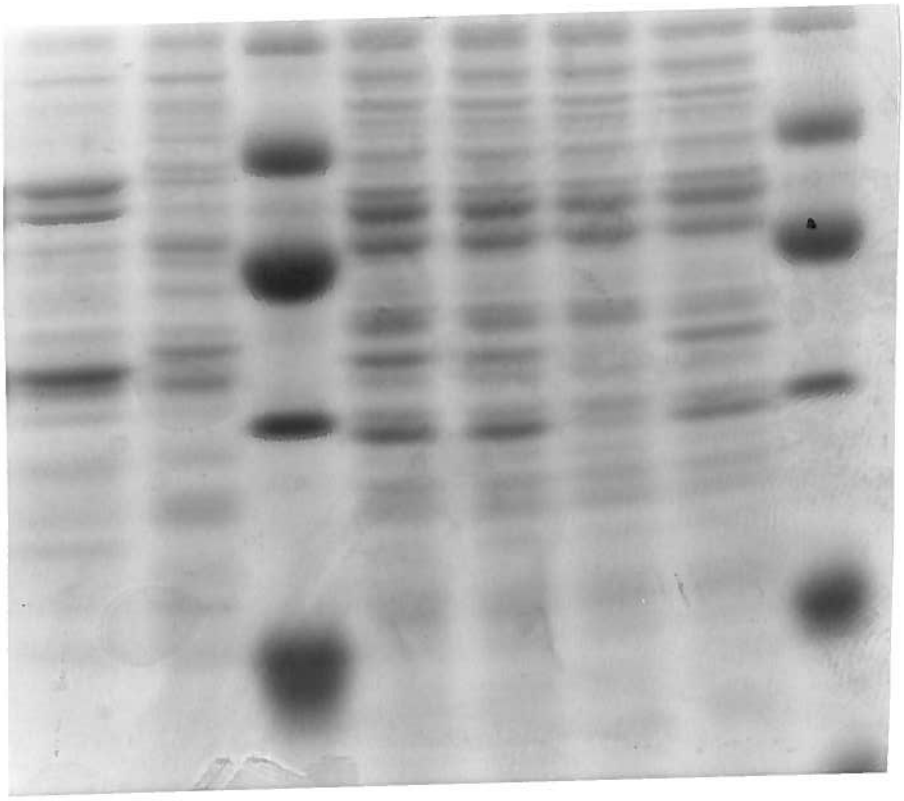

Figure 1.

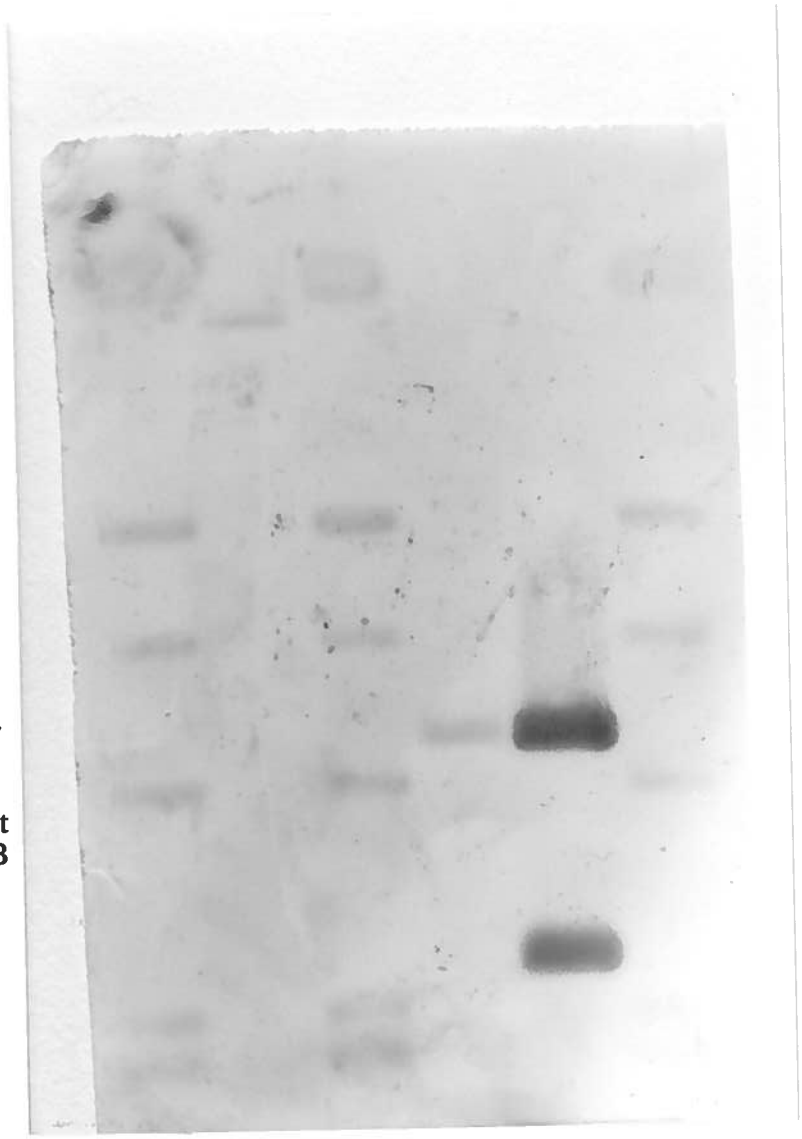

Figure 2.

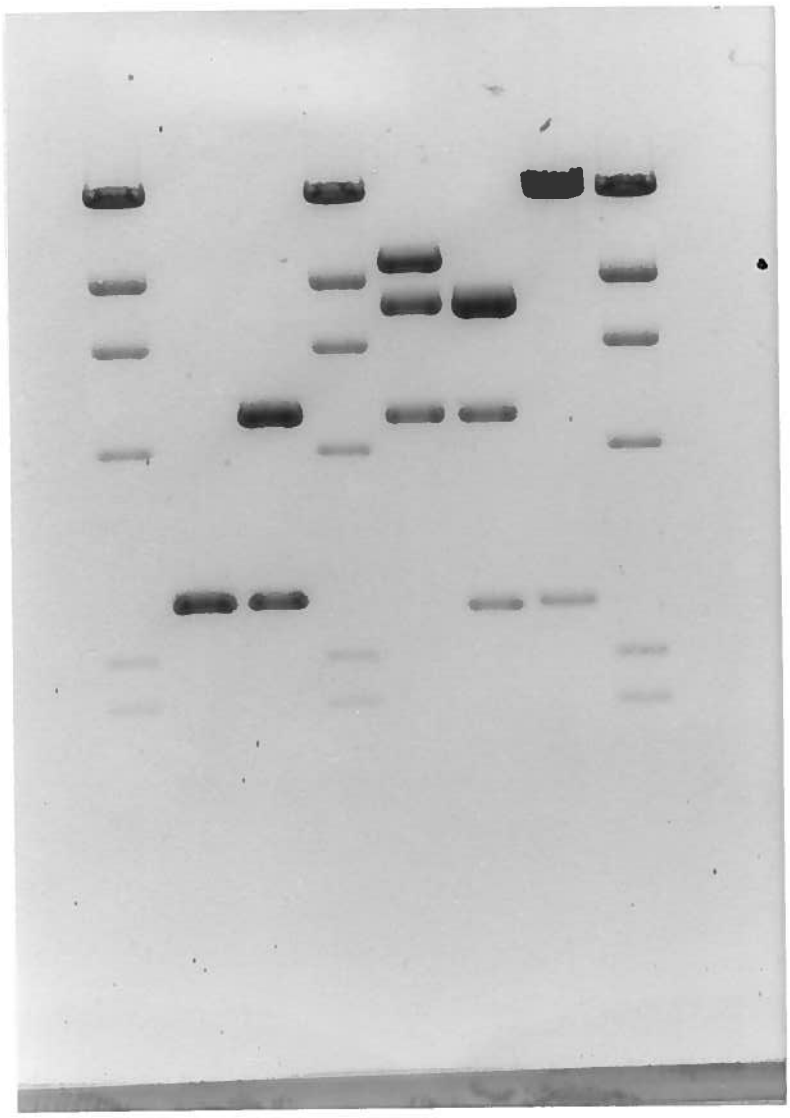

Figure 3.

Figure 4 A.

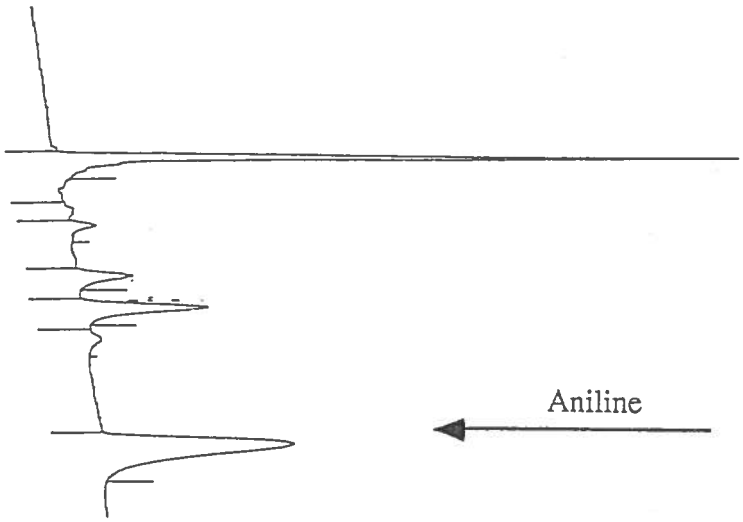

Figure 4 B.

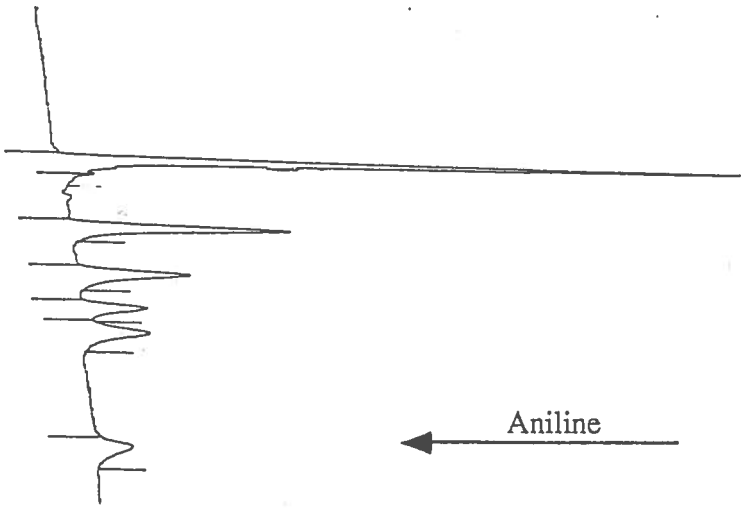

Figure 4. A) DH5α/pUC18 grown on aniline and glucose, B) DH5α/pSMT4 grown on aniline and glucose.

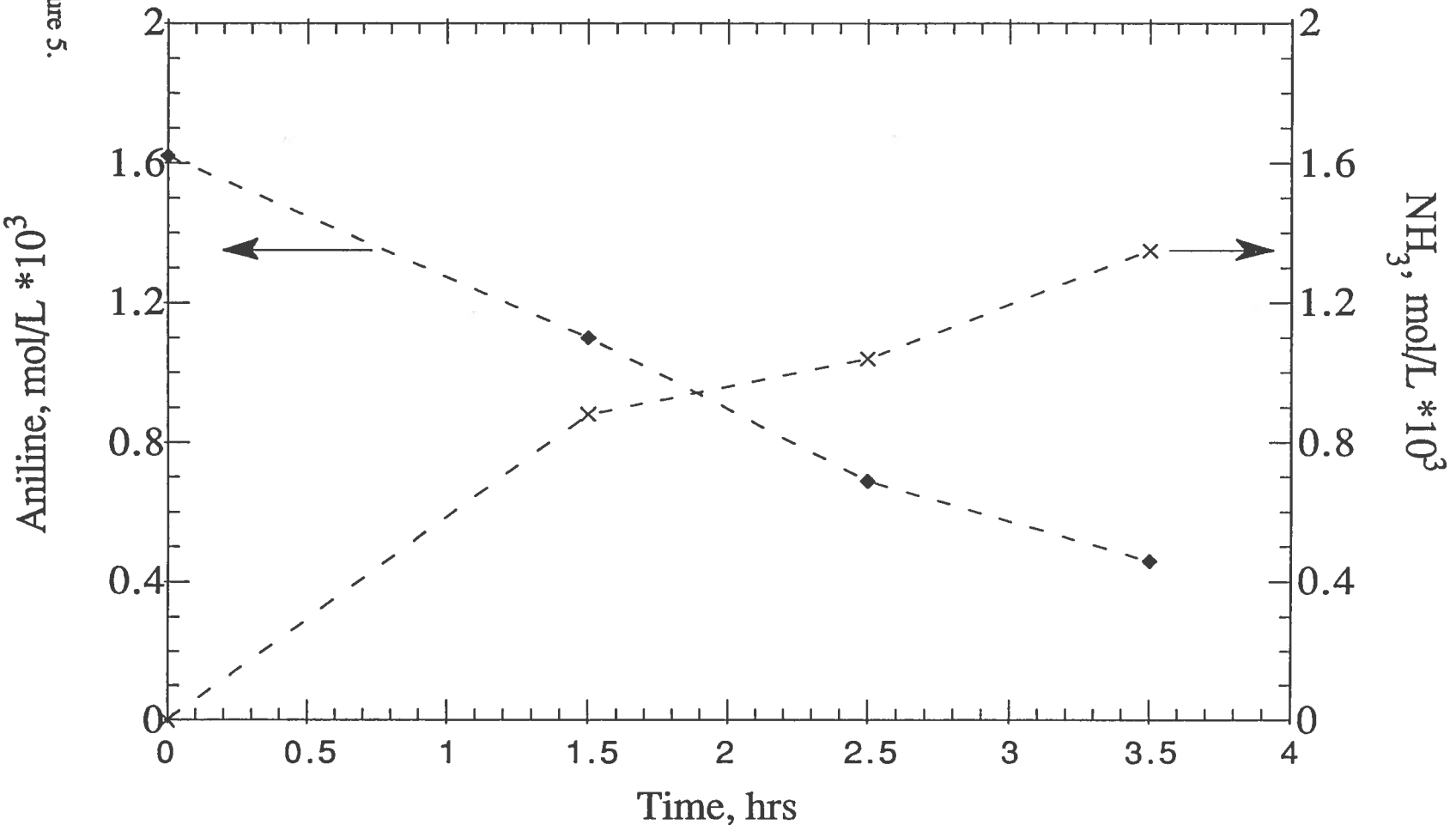

Figure 5. Aniline metabolism and ammonia release by *P. sp.* CIT1

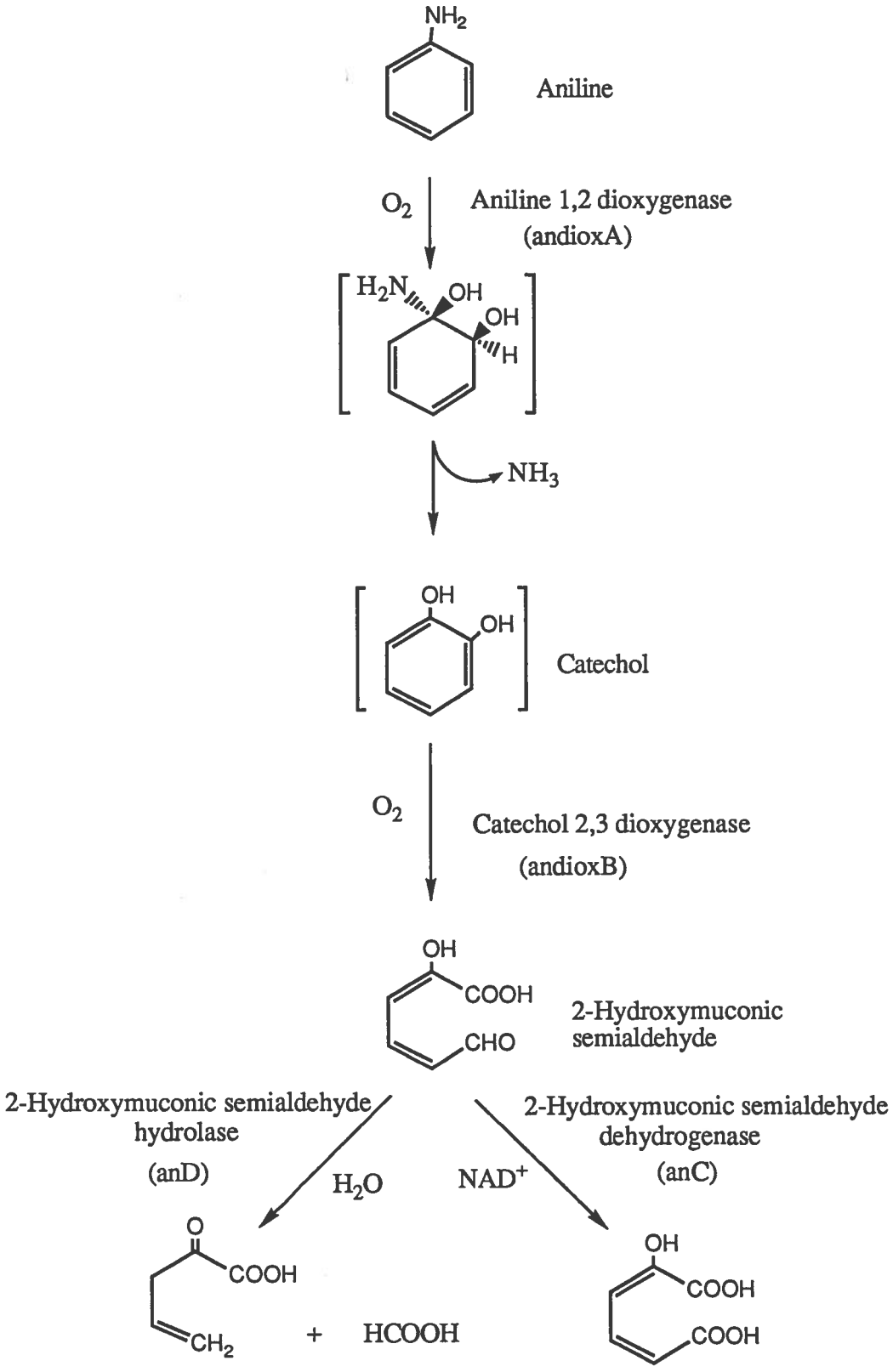

Figure 6. Proposed pathway for aniline metabolism in *P. sp.* s*P. sp.* CIT1